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TNF-related apoptosis-inducing ligand suppresses PRDX4 expression

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ABSTRACT

TNF-related apoptosis-inducing ligand (TRAIL) is currently considered a promising target for developing anti-cancer therapies. Accumulating evidences have now shown that oxidative stress is involved in the TRAIL-mediated cell death. The peroxiredoxins (PRDXs) are a ubiquitous family of proteins involved in protection against oxidative stress through the detoxification of cellular peroxides. Here we demonstrated that endogenous expression of PRDX4 was significantly decreased by TRAIL at the transcriptional level. In addition, overexpression of PRDX4 dramatically suppressed TRAIL-induced apoptosis. Taken together, these data for the first time suggested that TRAIL suppressed the PRDX4 gene at the transcriptional level and that downregulation of PRDX4 might facilitate cell death induced by TRAIL.

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL) is now considered to be a promising anticancer reagent, since its selective cytotoxicity in transformed tumor cells but not in normal cells [1,2]. TRAIL engages the extrinsic apoptotic pathway by binding to its membrane-bound death receptors (DR4 and DR5), an event followed by recruitment of intracellular adaptor molecule FADD and apoptosis initiator procaspase-8, forming the death-inducing signaling complex (DISC). When caspase-8 is cleaved, active fragment of caspase-8 is released into the cytosol where executioner of apoptosis, caspase-3 is activated for apoptosis [3]. The activation of caspase-8 by TRAIL induces the translocation of other cytosolic proapoptotic proteins to the mitochondria, causing a dissipation of the mitochondrial membrane potential [4]. Consequently, mitochondria release reactive oxygen species (ROS) and pro-apoptotic proteins into the cytoplasm thus inducing cellular and DNA damage [5]. The activation of pro-caspase-8 is believed to be dependent solely on proximity to other pro-caspase-8 units during recruitment to the DISC [6]. However, accumulating evidences suggested that whereas proximity is required for activation of pro-caspase-8, ROS could modulate the initiation of apoptosis signaling [7–10].

Peroxiredoxins (PRDXs) comprise an extended family of small antioxidant proteins which conserve a thioredoxin-dependent catalytic function that can contribute to cell protection from ROS [11,12]. PRDXs perform their protective antioxidant role in cells through their peroxidase activity whereby hydrogen peroxide, peroxynitrite and a wide range of organic hydroperoxides are reduced and detoxified [12]. At least six PRDXs were identified in mammalian cells [11,12]. In human, PRDX1–4, the typical 2-Cys subgroup, share two conserved motifs centered on Cys residues, which contain an additional 'resolving' cysteine near the C-terminus. PRDX5 differs because its C-terminal cysteine is not in the conserved position [13,14]. PRDX6 conserves only the Cys nearer the NH2-terminus, which is the catalytic site. PRDX are ubiquitously and abundantly expressed in the various tissues of the human body [15] and were reported to act in a mutually non-redundant and sometimes stress-specific fashion to protect human cells from oxidant injury [16].

ROS generation is one of the critical events in TRAIL-induced cancer cell death, but the role of PRDXs during TRAIL treatment has not been explored. We found that TRAIL specifically suppressed PRDX4 expression at the transcriptional level. In some cancer cells TRAIL did not cause cell death in spite of PRDX4 decrease. However, our data indicated that subtraction of PRDX4 during apoptosis, although not sufficient for initiation of apoptosis, is probably relevant in promoting cell death, since overexpression of PRDX4 dramatically suppressed TRAIL-induced apoptosis. Our data suggested that although PRDX4 suppression per se was not sufficient to induce cell death, its downregulation might facilitate cell death induced by TRAIL.

2. Materials and methods

2.1. Cell culture

The panel of cancer cells were maintained in DMEM (Sigma–Aldrich, Saint Louis, MO) supplemented with 10% FBS (Sigma–Aldrich).

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2.2. Reagents and antibodies

Human recombinant TRAIL and actinomycin D were purchased from Calbiochem (San Diego, CA) and Sigma–Aldrich (Saint Louis, MO), respectively. Western immunoblotting was performed using primary antibodies against PRDX1 (Novus Biologicals Inc., Littleton, CO), PRDX2 (GeneTex Inc., San Antonio, TX), PRDX3 (Novus Biologicals Inc.), PRDX4 (Abcam, Cambridge, MA), PRDX5 (Abnova, Walnut, CA), PRDX6 (Abcam), cytochrome c (BD Bioscience, San Jose, CA), poly ADP-ribose polymerase (PARP) (Cell Signaling Technology, Danvers, MA) or γ-tubulin (Sigma, Saint Louis, MO).

2.3. RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

RNA isolation and real-time RT-PCR were performed as previously reported [17]. 5′-CACAGCTGTTATGCCAGATG-3′ and 5′-ACT GAAAGCAATGATCTCCG-3′, 5′-AGATCATCGCGTTCAGCAAC-3′ and 5′-ATCCTCAGACAAGCGTCTGG-3′, 5′-GTCGCAGTCTCAGTGGATTC-3′ and 5′-AACAGCACACCGTAGTCTCG-3′, 5′-AACAGCTGTGATCGA TGGAG-3′ and 5′-TCAAGTCTGTCGCCAAAAGC-3′, 5′-CAAGAAGGG TGTGCTGTTTG-3′ and 5′-TAACACTCAGACAGGCCACC-3′, 5′-ATGCC TGTGACAGCTCGTGTG-3′ and 5′-TCTTCTTCAGGGATGGTTGG-3′ primer pairs were used to amplify PRDX1–6, respectively. For β-actin, the forward primer was 5′-GAGACCTTCAACACCCCAGCC-3′ and the reverse was 5′-GGATCTTCATGAGGTAGTCAG-3′. Results were normalized against those of β-actin.

2.4. Isolation of cytosolic and mitochondrial fractions

Cytosolic and mitochondrial fractions from HeLa cells were prepared as previously described [18].

2.5. Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-X100 and protease inhibitor cocktail (Sigma–Aldrich). Cell extract protein amounts were quantified using the BCA protein assay kit. Equivalent amounts of protein (25 μg) were separated using 12% SDS–PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, MA).

2.6. Generation of PRDX4 promoter luciferase constructs

The 5′-flanking region of human PRDX4 genomic DNA between −979 and +24 (+1 represents the translation start site, and the upstream nucleotide adjacent to the transcription start site is defined here as −1) was amplified by PCR from HeLa genomic DNA and subcloned into the reporter plasmid pGL4 (Promega, Madison, WI).

2.7. Luciferase assay

The luciferase activity was determined using the Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's instructions. All transfection experiments were repeated for three times in triplicate. Firefly (*Photinus pyralis*) luciferase activities normalized by *Renilla* (*Renilla reniformis*) activities are presented as fold induction relative to the normalized firefly luciferase activity in cells transfected with the pGL4 empty vector only, which was taken as 1.0.

2.8. Construction of PRDX4 plasmid and generation of HeLa cells stably overexpressing PRDX4

A cDNA encoding human PRDX4 was generated by polymerase chain reaction (PCR) from human brain cDNA library (Invitrogen, Carlsbad, CA) and subcloned into the eukaryotic expression plasmid pcDNA3 (pcDNA3-PRDX4). HeLa cells were transfected with pcDNA3-PRDX4 or an empty vector (pcDNA3-Flag) using Lipofectamine 2000 according to the protocol of the manufacturer. 48 h later, the cells were incubated in growth medium containing G418 (800 μg/ml, Life Technologies) to select stable clones. Four stable clones were selected based on the overexpression of PRDX4, which was confirmed by Western blotting.

2.9. Detection of cell death

For cell death assays, cells were washed twice in phosphate-buffered saline and then stained with Annexin V-FITC (Biovision, Mountainview, CA) and propidium iodide (PI, Sigma–Aldrich) according to the manufacturer's instructions. After staining with annexin V-FITC and PI, samples were analyzed by fluorescence-activated cell scanner (FACScan) flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

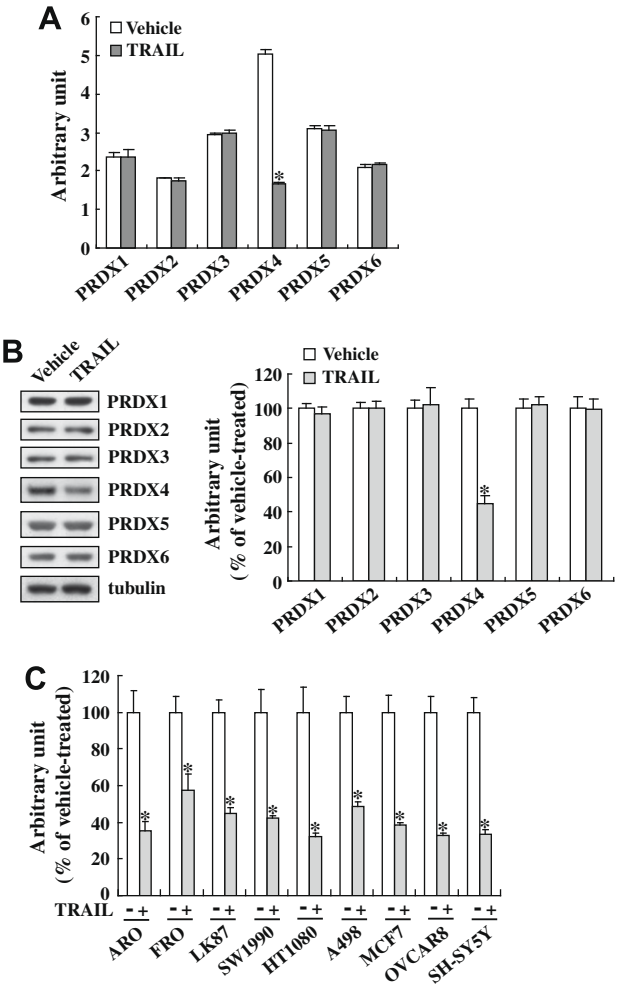

Fig. 1. The effect of TRAIL exposure on the PRDXs expression. (A) 8 h after cells had been treated with 1000 ng/ml TRAIL, total cellular RNA was extracted and real-time RT-PCR was performed. (B) HeLa cells were treated with 1000 ng/ml TRAIL for 8 h, total cellular proteins were isolated and Western blot was performed using indicated antibodies. A representative image was presented and the ratios *vs* that of vehicle-treated (normalized by tubulin) were graphed on the right of the image ($n = 3$). C, A panel of cancer cells was treated with 1000 ng/ml TRAIL for 8 h and PRDX4 expression was measured using real-time RT-PCR. All experiments were repeated three times, and each experimental condition was repeated in triplicate in each experiment. Data reported were average values ±S.D. of representative experiments. $^{*}P < 0.01$ as compared with vehicle-treated.

2.10. Measurement of intracellular ROS levels

The average level of intracellular ROS in thyroid cancer cells was evaluated in cells loaded with the redox-sensitive dye DCFH-DA (Molecular Probes, OR). Cells were washed twice in a phosphate-buffered saline (PBS) and stained in the dark for 30 min with 20 μM DCFH-DA and harvested. Cells were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength using a fluorescence spectrometer (HTS 7000, Perkin–Elmer, Boston, MA). A duplicate culture with the same treatments was used to determine the total protein levels. The ROS levels were expressed as arbitrary unit/mg protein, then as the percentage of control.

2.11. Statistics

The statistical significance of the difference was analyzed by ANOVA and post hoc Dunnett's test. Statistical significance was defined as $P < 0.05$. All experiments were repeated three times, and data were expressed as the mean ± S.D. (standard deviation) from a representative experiment.

3. Results

3.1. Suppression of PRDX4 mRNA/protein expressions by TRAIL exposure

We first performed real-time RT-PCR to study PRDXs mRNA expression regulation by TRAIL in HeLa cells. TRAIL had no obvious effect on the mRNA expression levels of PRDX1, PRDX2, PRDX3, PRDX5 and PRDX6 (Fig. 1A). However, incubation with TRAIL significantly decreased PRDX4 mRNA levels in HeLa cells (Fig. 1A). Western blot analysis also demonstrated a significant decrease in PRDX4 protein expression by TRAIL treatment (Fig. 1B). These results indicated that endogenous PRDX4 mRNA/protein expressions in HeLa cells were both suppressed by TRAIL exposure. To assess whether downregulation of PRDX4 by TRAIL was a cell-specific response, we investigated RPDX4 expression in a panel of cancer cells derived from various histological tissues and found that TRAIL inhibited PRDX4 expression in all cancer cells tested (Fig. 1C).

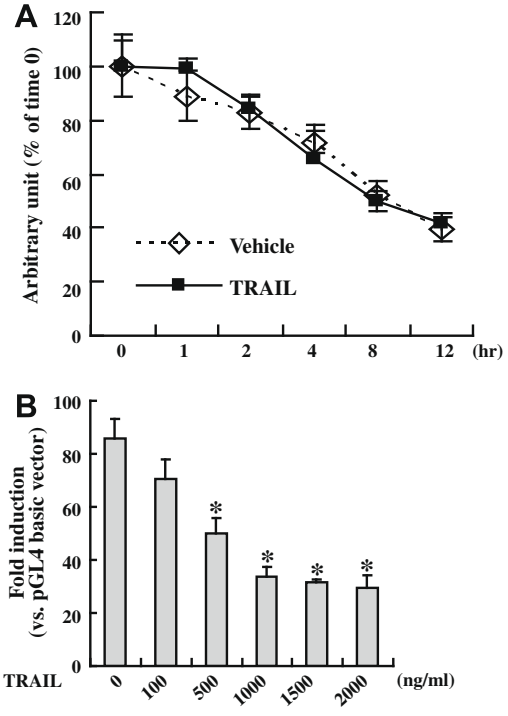

Fig. 2. Suppression of PRDX4 expression by TRAIL at the transcriptional level. (A) HeLa cells were pretreated with 20 μg/ml actinomycin D for 1 h, and incubated with vehicle or 1000 ng/ml TRAIL for an additional 8 h. Extracted RNAs were then processed to real-time RT-PCR. (B) 24 h after transfected with pPRDX4 promoter construct, HeLa cells were treated with indicated concentrations of TRAIL for 8 h and harvested for measuring luciferase activities. All experiments were repeated three times, and each experimental condition was repeated in triplicate in each experiment. Data reported were average values ±S.D. of representative experiments. $^{*}P < 0.01$ as compared with vehicle-treated.

3.2. Effect of TRAIL on PRDX4 mRNA stability

To examine whether the effect of TRAIL on PRDX4 mRNA decrease was due to decrease of PRDX4 mRNA stability or transcriptional suppression, we pretreated HeLa with vehicle or TRAIL for 8 h, then treated with 10 μg/ml actinomycin D for an additional indicated hours. PRDX4 mRNA in HeLa treated with vehicle (control) degraded approximately by 50% after 8 h treatment with actinomycin D (Fig. 2A). When HeLa cells were pretreated with TRAIL, PRDX4 mRNA degradation was not affected (Fig. 2A). These results suggested that TRAIL did not affect PRDX4 mRNA stability.

We next investigated whether TRAIL exposure can suppress the promoter activity of the PRDX4 gene in HeLa cells. pGL4-luc, in which the 5′-flanking region of human PRDX4 genomic DNA between −979 and +24 (+1 represents the transcription start site) was inserted upstream of a luciferase reporter, was transiently transfected into HeLa cells, and the luciferase activity was measured 8 h after exposure to TRAIL. TRAIL significantly decreased pPRDX4/−979 activity in a dose-dependent manner (Fig. 2B). These results suggested that TRAIL suppressed PRDX4 expression at the transcriptional level.

3.3. Downregulation of PRDX4 per se is not sufficient to initiate cell death

TRAIL-induced loss of PRDX4 occurred ahead of cleavage of PARP, the well known substrate of caspase-3 (Fig. 3A), as well as mitochondrial release of cytochrome c (Fig. 3B). In addition, TRAIL decreased PRDX4 expression in all cancer cells tested, while some types of cancer cells were resistant to TRAIL-induced cell demise (Fig. 3C). Collectively, these data suggested that TRAIL-mediated downregulation of PRDX4 per se might not be sufficient to initiate apoptosis.

3.4. Overexpression of PRDX4 inhibits TRAIL-induced apoptosis

Next, we examined the functional significance of TRAIL-induced downregulation of PRDX4 in apoptosis. To test whether PRDX4 overexpression could block the cell death by TRAIL, we employed HeLa cells stably transfected with PRDX4 (HeLa/PRDX4) and empty vector (HeLa/vector). Four different clones overexpressing several folds of PRDX4 were selected (Fig. 4A). The previously described TRAIL-mediated downregulation of PRDX4 expression was also observed in PRDX4 stable cells but with a lesser extent (Fig. 4B). Ectopic expression of PRDX4 significantly suppressed TRAIL-induced apoptosis (Fig. 4C), while had little effects on ROS generation (Fig. 4D), suggesting that downregulation of PRDX4 mediated by TRAIL might promote cell death independent of ROS production.

4. Discussion

TRAIL, a recently identified member of the tumor necrosis factor family, is capable of inducing apoptosis in various tumor cells [19,20]. Since it induces apoptosis in a variety of neoplastic cells while displaying minimal or absent cytotoxicity to most normal

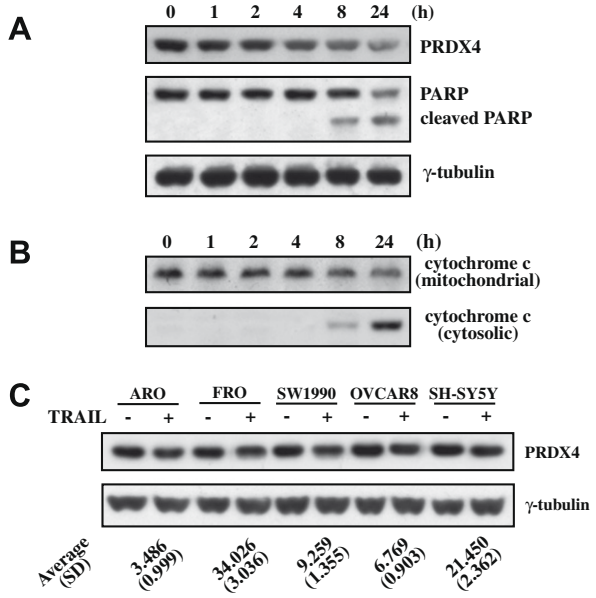

Fig. 3. Suppression of PRDX4 occurs ahead of initiation of apoptosis. (A) HeLa cells were treated with 1000 ng/ml TRAIL for the indicated time and Western blot was performed using indicated antibodies. (B) Cells were treated with 1000 ng/ml TRAIL for the indicated time, mitochondrial and cytosolic proteins were separated and release of mitochondria was analyzed. (C) A panel of cancer cells were treated with 1000 ng/ml TRAIL for 8 h and PRDX4 protein levels was analyzed. Apoptotic cells were noted at the bottom of the images.

cells, TRAIL is now considered a promising target for the development of anticancer therapies. Accumulating lines of evidence have now demonstrated that ROS is necessary to initiate TRAIL-mediated apoptosis in some carcinoma cells [7–10,21].

In eukaryotic cells, PRDXs not only act as antioxidants against ROS, but also influence processes such as apoptosis, cell-differentiation and proliferation [22]. PRDXs are ubiquitously and abundantly expressed in the various tissues of the human body [15]. These enzymes are produced at high levels in cells: they are the second or third most abundant protein in erythrocytes and compose 0.1–0.8% of the soluble protein in other mammalian cells [23]. Many organisms produce more than one isoform of PRDX, including at least six PRDXs identified in mammalian cells, which are distributed differentially within cells: PRDX1, 2, and 4 are localized to the cytosol; PRDX3 is restricted to mitochondria; PRDX4 is located in ER and also extracellularly secreted; and PRDX5 is located in mitochondria and peroxisomes [11,12]. PRDXs contain a reactive Cys in a conserved region near the N-terminus, and this catalytic Cys residue forms cystein–sulfenic acid as a reaction intermediated during the reduction of peroxide. All the PRDXs exhibit peroxidase activity dependent on reduced forms of thioredoxin and/or glutathione. PRDX1–4 has an additional Cys at a conserved position near the C-terminus. PRDXs are not redundant functionally and individual PRDXs may protect against different stresses [16]. In this context, it is interesting to note that only PRDX4 expression was altered during TRAIL exposure. Mammals possess six PRDX isoforms with cellular locations including the cytosol (PRDX1, 2, 6), nucleus (PRDX1), motochondria (PRDX3, 6) and peroxisomes (PRDX5). PRDX4 has been described as both an endoplasmic reticulum protein and as a secreted protein [24–26]. TRAIL significantly decreased PRDX4 mRNA expression level in multiple cancer cells derived from different histology. In addition, TRAIL also decreased PRDX4 protein expression level. TRAIL did not affect PRDX4 mRNA stability and PRDX4 gene promoter activity was inhibited by TRAIL, suggesting that PRDX4 expression was suppressed at the transcriptional level. While PRDX4 downregulation per se may not be sufficient to induce cell death, PRDX4 suppression has some favorable clinical efficacy in the cancer therapy, since overexpression significantly suppressed TRAIL-mediated cell death. In addition, PRDX4 overexpression had little effects on ROS generation, suggesting that the effects witnessed following PRDX4 overexpression may be attributable to its presence helping to alleviate the after-effects of oxidative insult, consisting with previous report [26].

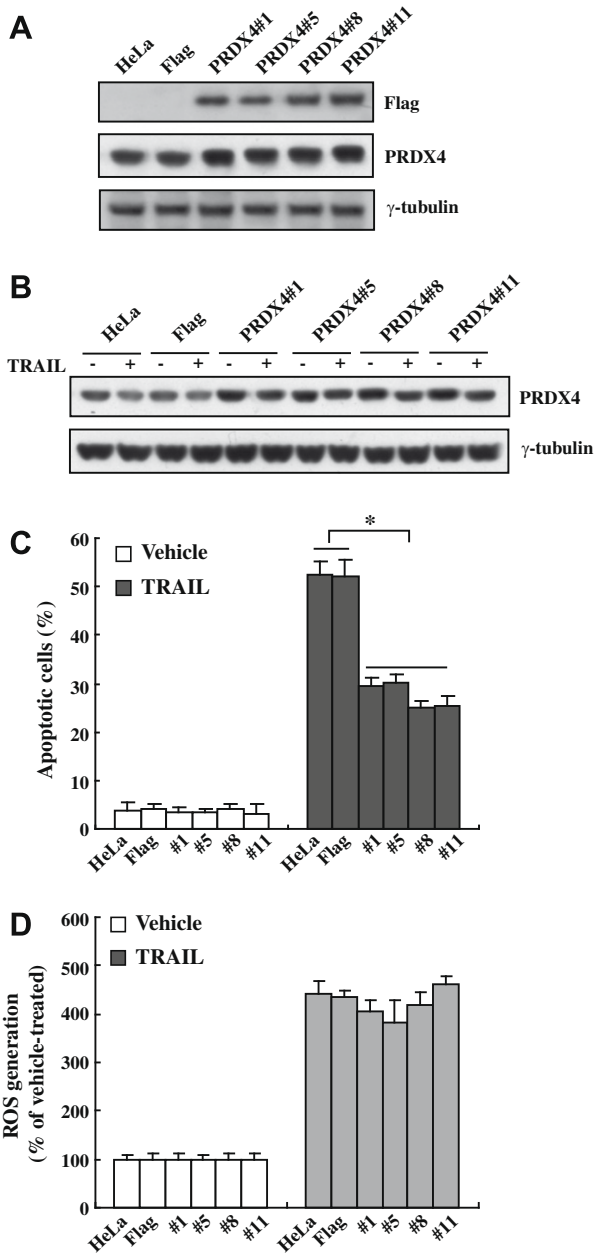

Fig. 4. Overexpression of PRDX4 suppressed TRAIL-induced apoptosis. (A) Total protein isolated from parent HeLa, Flag stable or PRDX4 stable cells were subjected to Western blot. (B–D) Cells were treated with 1000 ng/ml TRAIL for 24 h, and PRDX4 levels (B), apoptotic cells (C) and ROS generation (D) were analyzed. All experiments were repeated three times, and each experimental condition was repeated in triplicate in each experiment. Data reported were average values ±S.D. of representative experiments. $^{*}P < 0.01$.

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